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(51) International Patent Classification ⁶ : C12N 15/85, C12Q 1/68, G01N 33/50, C12N 9/64	A1	(11) International Publication Number: WO 97/41242 (43) International Publication Date: 6 November 1997 (06.11.97)
(21) International Application Number: PCT/US97/06504 (22) International Filing Date: 17 April 1997 (17.04.97) (30) Priority Data: 60/015,813 18 April 1996 (18.04.96) US (71) Applicant: GERON CORPORATION [US/US]; 200 Constitution Drive, Menlo Park, CA 94025 (US). (72) Inventors: CAMPISI, Judith; 1 Roble Road, Berkeley, CA 94705 (US). TESTORI, Alessandro; 2208 Martin Luther King Jr. Way #5, Berkeley, CA 94704 (US). (74) Agents: STORELLA, John, R. et al.; Townsend and Townsend and Crew L.L.P., 8th floor, Two Embarcadero Center, San Francisco, CA 94111 (US).		(81) Designated States: AU, CA, CN, JP, KR, MX, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SENESCENCE RESPONSIVE TRANSCRIPTIONAL ELEMENT (57) Abstract Recombinant polynucleotides have expression control sequences that have a senescence responsive element and a minimal promoter, and which are operatively linked to a heterologous nucleotide sequence. The molecules are useful for achieving high levels of expression of genes in senescent cells. Methods of inhibiting expression of genes in senescent cells also are provided.		

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SENESCENCE RESPONSIVE TRANSCRIPTIONAL ELEMENT

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BACKGROUND OF THE INVENTION

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This invention relates to the field of regulation of gene expression.

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Animal cells undergo replicative senescence -- age-dependent decrements in function and integrity. One aspect of replicative senescence is that cells have a finite replicative life-span. After undergoing a limited number of mitotic divisions, they can no longer divide. Upon replicative senescence, cells stably arrest growth with a G1 DNA content, irreversibly losing the ability to enter S phase in response to physiologic mitogens. Another aspect of replicative senescence is that senescent cells acquire altered functions, resembling terminally differentiated cells. A third aspect of replicative senescence is that senescent cells acquire resistance to apoptotic cell death.

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Senescent cells exhibit altered gene expression compared with pre-senescent cells. In particular, several genes increase expression. For example, senescent human fibroblasts show a marked increase in interstitial collagenase mRNA and protein. Burke et al., *Exp. Gerontol.*, (1994) 29:37-53. Senescent cells also exhibit increased expression of stromelysin, the adhesion molecule ICAM, and certain inflammatory

mediators, such as IL-1. Other genes, such as certain mitogen inducible genes and IL-6, are repressed in senescent cells. Altered gene expression manifests itself at the tissue and organismic level.

For example, collagenase breaks down collagen, particularly, the fibrillar collagens (types I, II and III). The extracellular matrix is made of a complex network of proteins and proteoglycans, including collagen, that maintain the integrity of tissues. J. D'Armiento et al., *Cell*, (1992) 71:955-961. The turnover of these molecules is effected by, among other things, matrix metalloproteinases such as interstitial collagenase. Interstitial collagenase has been implicated in osteoarthritis, periodontal lesions, bullous skin lesions, cancer invasion and a variety of liver and lung diseases. For example, injection of collagenase into the joints of mice results in lesions similar to osteoarthritis. P.M. van der Kraan, *J. Exp. Path.*, (1990) 71:19-31. Emphysema is characterized by disruption of the alveolar walls and coalescence of the alveolar spaces. Mice made transgenic for a construct that expresses the collagenase gene in lung cells exhibited these symptoms. D'Armiento et al., *supra*. Epithelial organs, including the skin, have an outer epidermal layer, a basement membrane and a layer of dermis comprising a stroma that includes fibroblasts. Skin, in particular, is composed of stratified, squamous, keratinized epithelium, called the epidermis. Aged skin contains a greater number of senescent cells than young skin. Aged skin tends to be wrinkled, dry and scaly. Mice made transgenic for a construct that expresses the collagenase gene in skin took on characteristics of aged skin: Their skin was dry, wrinkled and scaly, and exhibited acanthosis, hyperkeratosis and epidermal hyperplasia. Also, the transgenic mice demonstrated increased susceptibility to tumorigenesis. J. D'Armiento et al., *Molec. and Cell Biol.*, (1995) 15:5732-39.

Certain premature aging syndromes, such as Werner syndrome, are characterized by premature cellular senescence. Persons with Werner syndrome show hair and dermal thinning, atherosclerosis, osteoporosis and increased incidence of cancer.

Replicative senescence and its regulation are an increasingly active area of study. Genetic markers of senescence and methods of expressing genes in senescent cells or altering expression of genes in senescent cells would constitute advances in the art.

SUMMARY OF THE INVENTION

Nucleotide sequences between nucleotides -35 and -134, and, more specifically, between nucleotides -35 and -100 of the human interstitial collagenase gene promoter (SEQ ID NO:1) have been found to contain elements responsible for much of the increased transcription of collagenase in senescent cells. These sequences are called the senescence responsive element, or "SnRE." Removal of the senescence responsive element from the collagenase promoter eliminates increased transcription from the promoter in senescent cells.

The interstitial collagenase gene promoter contains three known transcriptional regulatory elements: a PEA-3 (or ets) element at position -89 to -82; a CdxA element at -81 to -75; and an AP-1 element at position -72 to -66. Mutating the AP-1 site abolishes transcription in senescent cells. Mutating the PEA-3 site decreases promoter activity by about 40%. However, eliminating sequences upstream of the AP-1 site, e.g., upstream of nucleotide -77, also abolishes promoter activity.

Placing a senescence responsive element upstream of other promoters in a senescent cell results in increased transcription of genes to which it is operatively linked.

Accordingly, this invention provides a recombinant polynucleotide comprising an expression control sequence operatively linked to a heterologous nucleotide sequence, the expression control sequence comprising (1) a senescence responsive element and (2) a minimal promoter positioned downstream of the senescence responsive element, wherein the senescence responsive element up-regulates transcription of the heterologous nucleotide sequence in a senescent cell. In one embodiment, the minimal promoter is heterologous with respect to the senescence responsive element, e.g., it is not the interstitial collagenase gene minimal promoter. In another embodiment, the SnRE comprises nucleotides -89 to -66 of the interstitial collagenase gene promoter (5'-gaggatgta taaagcatga gtca -3' (SEQ ID: NO:1).

In another aspect, this invention provides a method for expressing a heterologous nucleotide sequence in a senescent cell comprising the step of providing the cell with a recombinant polynucleotide comprising an expression control sequence operatively linked to a heterologous nucleotide sequence, the expression control sequence comprising (1) a senescence responsive element and (2) a minimal promoter positioned downstream of the senescence responsive element, wherein the senescence responsive

element up-regulates transcription of the heterologous nucleotide sequence in a senescent cell; and culturing the cell under conditions for expression.

5 In another aspect, this invention provides an isolated recombinant host cell comprising a recombinant polynucleotide comprising an expression control sequence operatively linked to a heterologous nucleotide sequence, the expression control sequence comprising (1) a senescence responsive element and (2) a minimal promoter positioned downstream of the senescence responsive element, wherein the senescence responsive element up-regulates transcription of the heterologous nucleotide sequence in a senescent cell.

10 In another aspect, this invention provides a transgenic non-human animal having a germ cell that comprises a recombinant polynucleotide comprising an expression control sequence operatively linked to a heterologous nucleotide sequence, the expression control sequence comprising (1) a senescence responsive element and (2) a minimal promoter positioned downstream of the senescence responsive element, wherein the
15 senescence responsive element up-regulates transcription of the heterologous nucleotide sequence in a senescent cell.

In another aspect, this invention provides methods for monitoring senescence in a cell, cell population, tissue, organ or organism. In one embodiment, the method comprises the steps of culturing, under conditions for expression, a recombinant
20 host cell of this invention; detecting or measuring the amount of expression of the reporter gene by the cell; and determining whether the measured amount is different than the amount expected in a pre-senescent cell. The difference between the measured amount and the expected amount reflects the degree to which the cell is senescent. In other embodiments, the methods comprise measuring the amount of, or detecting,
25 expression of the reporter gene in a cell population, tissue, organ or organism comprising recombinant cells of the invention, and determining whether the amount of expression is different than an expected amount in the sample in a pre-senescent state.

In another aspect, this invention provides a method for determining whether a compound alters the activity of a senescence responsive element in a cell. The
30 method comprises the steps of culturing, under conditions for expression; a recombinant cell of this invention; contacting the cell with the compound; measuring the amount of expression of the reporter polypeptide; and determining whether the measured amount is different than the amount expected from a recombinant host cell to which no compound

has been administered. A difference between the measured amount and the expected amount indicates that the compound alters the activity of the senescence responsive element in the cell.

5 In another aspect, this invention provides a compound identified by the aforesaid method.

In another aspect, this invention provides a polynucleotide probe that specifically hybridizes with a contiguous nucleotide sequence within nucleotides -35 to -134 of the interstitial collagenase gene promoter, or with a sequence complementary to a contiguous nucleotide sequence within nucleotides -35 to -134 of the promoter.

10 In another aspect, this invention provides an inhibitory polynucleotide comprising an antisense sequence of at least 7 nucleotides that specifically hybridizes to a nucleotide sequence within nucleotides -35 to -134 of the interstitial collagenase gene promoter, or the complement of the promoter sequence, wherein the inhibitory polynucleotide inhibits transcription of nucleotide sequences operatively linked to
15 expression control sequences comprising a senescence responsive element.

In another aspect, this invention provides a method of inhibiting in a cell the expression of a nucleotide sequence operatively linked to an expression control sequence that comprises a senescence responsive element, the method comprising the step of providing the cell with an inhibitory polynucleotide of this invention.

20 In another aspect this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a prophylactically or therapeutically effective amount of: (a) a recombinant polynucleotide comprising an expression control sequence operatively linked to a heterologous nucleotide sequence, the expression control sequence comprising (1) a senescence responsive element and (2) a minimal promoter
25 positioned downstream of the senescence responsive element, wherein the senescence responsive element up-regulates transcription of the heterologous nucleotide sequence in a senescent cell; (b) a recombinant polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence coding for the expression an inhibitory polynucleotide comprising nucleotides in a sequence substantially identical to or
30 substantially complementary to a contiguous sequence of 6 nucleotides within nucleotides -35 to -134 of SEQ ID NO:1, wherein the inhibitory polynucleotide inhibits transcription of nucleotide sequences operatively linked to expression control sequences comprising the senescence responsive element; or (c) an inhibitory polynucleotide comprising an

antisense sequence of at least 7 nucleotides that specifically hybridizes to a nucleotide sequence within nucleotides -35 to -134 of the interstitial collagenase gene promoter, or the complement of the promoter sequence, wherein the inhibitory polynucleotide inhibits transcription of nucleotide sequences operatively linked to expression control sequences comprising a senescence responsive element.

In another aspect this invention provides methods of prophylactically or therapeutically treating a senescent condition in a subject. Therapeutic agents can be administered in the pharmaceutical preparations of this invention.

A method of treating a senescent condition associated with the activity of a senescence responsive element comprises the step of inhibiting the expression of a gene operatively linked to an expression control sequence that includes the senescence responsive element. Inhibiting expression of the gene provides a treatment of the senescent condition. Inhibiting expression can involve, for example, providing an inhibitory polynucleotide of the invention.

A method of prophylactically or therapeutically treating a senescent condition associated with under-expression of a gene comprises the step of providing cells of the subject with a recombinant polynucleotide comprising expression control sequences comprising a senescence responsive element operatively linked to the gene. Expression of the gene provides a treatment of the senescent condition.

In particular, this invention provides prophylactic and therapeutic methods of treating aged epithelium, cancers of the epithelium, and inflammatory diseases of the aged, such as osteoarthritis, Alzheimer's disease and atherosclerosis.

In another aspect this invention provides a method of determining whether a sample comprises a compound that binds to a senescence responsive element comprising contacting the sample with a polynucleotide probe of the invention; and determining whether a compound has bound to the probe. Binding indicates that the sample contains a compound that binds to the senescence responsive element.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Human interstitial collagenase is over-expressed in senescent human fibroblasts (WI-38) due to increased transcription initiation. The 3470 bp collagenase promoter driving the luciferase reporter (pGL-3470) was transfected into pre-senescent and senescent cells and reporter activity was measured 72 h later. Senescent

cells expressed 15- to 20-fold more luciferase activity than pre-senescent cells. The promoter-less reporter vector (pGL3) was inactive in both cell types.

Fig. 2: The region between -35 and -100 (1 = start of transcription) accounts for the largest increase in promoter activity in senescent cells. Several 5' deletions in the collagenase promoter in pGL-3470 were made, and the deletion vectors were transfected into pre-senescent and senescent cells. Deletion up to -134 resulted in a 3-4 fold reduction in promoter activity in senescent cells; deletion up to -34 resulted in a 20- to 25-fold reduction in promoter activity in senescent cells. All deletion constructs showed 10- to 15-fold less activity in pre-senescent cells.

Fig. 3: The promoter region -35 to -134 contains the major determinant of the enhanced transcription in senescent cells. Deletion of this region (PGL 522 Δ 34/134 construct) resulted in a drastic decrease in the promoter activity in senescent cells.

Fig. 4: The nucleotide sequence of nucleotides -134 to -34 of the human interstitial collagenase promoter (SEQ ID NO:1).

Fig. 5: The nucleotide sequence of the 5' un-transcribed regions of the human interstitial collagenase gene, including (SEQ ID NO:2). This sequence is from Angel et al., *Molec. and Cell Biol.*, (1987) 7:2256-66.

DETAILED DESCRIPTION OF THE INVENTION

I. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton et al., *Dictionary of Microbiology and Molecular Biology* (2d ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

"Polynucleotide" refers to a polymer composed of nucleotide units (ribonucleotides, deoxyribonucleotides, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof) linked via phosphodiester bonds, related naturally occurring structural variants, and synthetic non-naturally occurring

analogous thereof. Thus, the term includes nucleotide polymers in which the nucleotides and the linkages between them include non-naturally occurring synthetic analogs, such as, for example and without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide nucleic acids ("PNAs"), and the like. Such polynucleotides can be synthesized, for example, using an automated DNA synthesizer. "Nucleic acid" typically refers to large polynucleotides. "Oligonucleotide" typically refers to short polynucleotides, generally no greater than about 50 nucleotides. It will be understood that when a nucleotide sequence is represented by a DNA sequence (i.e., A, T, G, C), this also includes an RNA sequence (i.e., A, U, G, C) in which "U" replaces "T."

Conventional notation is used herein to describe polynucleotide sequences: the left-hand end of a single-stranded polynucleotide sequence is the 5'-end; the left-hand direction of a double-stranded polynucleotide sequence is referred to as the 5'-direction. The direction of 5' to 3' addition of nucleotides to nascent RNA transcripts is referred to as the transcription direction. The DNA strand having the same sequence as an mRNA is referred to as the "coding strand"; sequences on the DNA strand having the same sequence as an mRNA transcribed from that DNA and which are located 5' to the 5'-end of the RNA transcript are referred to as "upstream sequences"; sequences on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the coding RNA transcript are referred to as "downstream sequences."

"Recombinant polynucleotide" refers to a polynucleotide having sequences that are not naturally joined together. An amplified or assembled recombinant polynucleotide may be included in a suitable vector, and the vector can be used to transform a suitable host cell. A host cell that comprises the recombinant polynucleotide is referred to as a "recombinant host cell." The gene is then expressed in the recombinant host cell to produce, e.g., a "recombinant polypeptide." A recombinant polynucleotide may serve a non-coding function (e.g., promoter, origin of replication, ribosome-binding site, etc.) as well. Appropriate unicellular hosts include any of those routinely used in expressing eukaryotic or mammalian polynucleotides, including, for example, prokaryotes, such as *E. coli*; and eukaryotes, including for example, fungi, such as yeast; and mammalian cells, including insect cells (e.g., Sf9) and animal cells such as CHO, R1.1, B-W, L-M, African Green Monkey Kidney cells (e.g. COS 1, COS 7, BSC 1, BSC 40 and BMT 10) and cultured human cells.

Terms used to describe sequence relationships between two or more nucleotide sequences or amino acid sequences include "reference sequence," "selected from," "comparison window," "identical," "percentage of sequence identity," "substantially identical," "complementary," and "substantially complementary."

5 A "reference sequence" is a defined sequence used as a basis for a sequence comparison and may be a subset of a larger sequence, e.g., a complete cDNA, protein, or gene sequence.

Because two polynucleotides or polypeptides each may comprise (1) a sequence (i.e., only a portion of the complete polynucleotide or polypeptide sequence)
10 that is similar between the two polynucleotides, or (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides or polypeptides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

15 A "comparison window" refers to a conceptual segment of typically at least 12 consecutive nucleotide or 4 consecutive amino acid residues that is compared to a reference sequence. The comparison window frequently is at least 15 or at least 25 nucleotides in length or at least 5 or at least 8 amino acids in length. The comparison window may comprise additions or deletions (i.e., gaps) of about 20 percent or less as
20 compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerized implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI) or by
25 inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by any of the various methods is selected.

A subject nucleotide sequence or amino acid sequence is "identical" to a reference sequence if the two sequences are the same when aligned for maximum correspondence over the length of the nucleotide or amino acid sequence.

30 The "percentage of sequence identity" between two sequences is calculated by comparing two optimally aligned sequences over a comparison window, determining the number of positions at which the identical nucleotide or amino acid occurs in both sequences to yield the number of matched positions, dividing the number of matched

positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. Unless otherwise specified, the comparison window used to compare two sequences is the length of the shorter sequence.

5 When percentage of sequence identity is used in reference to polypeptides it is recognized that residue positions that are not identical often differ by conservative amino acid substitutions, where amino acids residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in
10 conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of
15 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., according to known algorithm. See, e.g., Meyers & Miller, *Computer Applic. Biol. Sci.*, 4: 11-17 (1988); Smith & Waterman, *Adv. Appl. Math.* 2: 482 (1981); Needleman & Wunsch, *J. Mol. Biol.* 48: 443 (1970); Pearson & Lipman, *Proc. Natl. Acad. Sci. USA* 85: 2444 (1988); Higgins & Sharp *Gene*, 73: 237-244 (1988); Higgins & Sharp, *CABIOS* 5: 151-153 (1989); Corpet *et al.*, *Nucleic Acids Research* 16: 10881-90 (1988); Huang *et al.*, *Computer Applications in the Biosciences* 8: 155-65 (1992); and Pearson *et al.*, *Methods in Molecular Biology* 24: 307-31 (1994). Alignment is also often performed by inspection and manual alignment.

25 A subject nucleotide sequence or amino acid sequence is "substantially identical" to a reference sequence if the subject amino acid sequence or nucleotide sequence has at least 80% sequence identity over a comparison window. Thus, sequences that have at least 85% sequence identity, at least 90% sequence identity, at least 95% sequence identity, at least 98% sequence identity or at least 99% sequence
30 identity with the reference sequence are also "substantially identical." Two sequences that are identical to each other are, of course, also "substantially identical".

"Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be

described as complementary, and furthermore, the contact surface characteristics are complementary to each other. A first polynucleotide is complementary to a second polynucleotide if the nucleotide sequence of the first polynucleotide is identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide.

5 Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

A nucleotide sequence is "substantially complementary" to a reference nucleotide sequence if the sequence complementary to the subject nucleotide sequence is substantially identical to the reference nucleotide sequence.

10 "Expression control sequence" refers to a nucleotide sequence in a polynucleotide that regulates the expression (transcription and/or translation) of a nucleotide sequence operatively linked to it. "Operatively linked" refers to a functional relationship between two parts in which the activity of one part (e.g., the ability to regulate transcription) results in an action on the other part (e.g., transcription of the
15 sequence). Expression control sequences can include, for example and without limitation, sequences of promoters (e.g., inducible or constitutive), enhancers, transcription terminators, a start codon (i.e., ATG), splicing signals for introns, and stop codons.

"Expression vector" refers to a vector comprising a recombinant
20 polynucleotide comprising expression control sequences operatively linked to a nucleotide sequence to be expressed. An expression vector comprises sufficient *cis*-acting elements for expression; other elements for expression can be supplied by the host cell or *in vitro* expression system. Expression vectors include all those known in the art, such as cosmids, plasmids (e.g., naked or contained in liposomes) and viruses that incorporate
25 the recombinant polynucleotide.

"Encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (i.e., rRNA, tRNA and mRNA) or a
30 defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene encodes a protein if transcription and translation of mRNA produced by that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided

in sequence listings, and non-coding strand, used as the template for transcription, of a gene or cDNA can be referred to as encoding the protein or other product of that gene or cDNA. Unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may include introns.

"Allelic variant" refers to any of two or more polymorphic forms of a gene occupying the same genetic locus. Allelic variations arise naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. "Allelic variant" also refers to polymorphisms in non-coding sequences at a genetic locus and cDNAs derived from mRNA transcripts of genetic allelic variants, as well as the proteins encoded by them.

"Hybridizing specifically to" or "specific hybridization" or "selectively hybridize to," refers to the binding, duplexing, or hybridizing of a polynucleotide preferentially to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (e.g., total cellular) DNA or RNA.

"Stringent conditions" refers to conditions under which a probe will hybridize preferentially to its target subsequence, and to a lesser extent to, or not at all to, other sequences. "Stringent hybridization" and "stringent hybridization wash conditions" in the context of polynucleotide hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of polynucleotides is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes* part I chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe.

An example of stringent hybridization conditions for hybridization of complementary polynucleotides which have more than 100 complementary residues on a

filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42° C, with the hybridization being carried out overnight. An example of highly stringent wash conditions is 0.15 M NaCl at 72° C for about 15 minutes. An example of stringent wash conditions is a 0.2X SSC wash at 65° C for 15 minutes (see, Sambrook et al. for a description of SSC buffer). Often, a high stringency wash is preceded by a low stringency wash to remove background probe signal. An example medium stringency wash for a duplex of, e.g., more than 100 nucleotides, is 1x SSC at 45° C for 15 minutes. An example low stringency wash for a duplex of, e.g., more than 100 nucleotides, is 4-6x SSC at 40° C for 15 minutes. In general, a signal-to-noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization.

A first sequence is an "antisense sequence" with respect to a second sequence if a polynucleotide whose sequence is the first sequence specifically hybridizes with a polynucleotide whose sequence is the second sequence.

"Reporter gene" refers to a gene that codes for the expression of a polynucleotide or polypeptide capable of acting as a label. Reporter genes include those encoding enzymes such as β -galactosidase, luciferase, secreted alkaline phosphatase and fluorescent proteins. e.g., green fluorescent protein.

"Primer" refers to a polynucleotide that is capable of specifically hybridizing to a designated polynucleotide template and providing a point of initiation for synthesis of a complementary polynucleotide. Such synthesis occurs when the polynucleotide primer is placed under conditions in which synthesis is induced, i.e., in the presence of nucleotides, a complementary polynucleotide template, and an agent for polymerization such as DNA polymerase. A primer is typically single-stranded, but may be double-stranded. Primers are typically deoxyribonucleic acids, but a wide variety of synthetic and naturally occurring primers are useful for many applications. A primer is complementary to the template to which it is designed to hybridize to serve as a site for the initiation of synthesis, but need not reflect the exact sequence of the template. In such a case, specific hybridization of the primer to the template depends on the stringency of the hybridization conditions. Primers can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as detectable moieties.

"Probe" refers to a polynucleotide that is capable of specifically hybridizing to a designated sequence of another polynucleotide. A probe specifically

hybridizes to a target complementary polynucleotide, but need not reflect the exact complementary sequence of the template. In such a case, specific hybridization of the probe to the target depends on the stringency of the hybridization conditions. Probes can be labeled with, e.g., chromogenic, radioactive, or fluorescent moieties and used as
5 detectable moieties.

"Detecting" refers to determining the presence, absence, or amount of an analyte in a sample, and can include quantifying the amount of the analyte in a sample or per cell in a sample.

"Detectable moiety" or a "label" refers to a composition detectable by
10 spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P , ^{35}S , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin-streptavidin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or polynucleotides with a sequence complementary to a target. The detectable moiety often
15 generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantitate the amount of bound detectable moiety in a sample. The detectable moiety can be incorporated in or attached to a primer or probe either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., incorporation of radioactive nucleotides, or biotinylated nucleotides that are recognized by streptavidin.
20 The detectable moiety may be directly or indirectly detectable. Indirect detection can involve the binding of a second directly or indirectly detectable moiety to the detectable moiety. For example, the detectable moiety can be the ligand of a binding partner, such as biotin, which is a binding partner for streptavidin, or a nucleotide sequence, which is the binding partner for a complementary sequence, to which it can specifically hybridize.
25 The binding partner may itself be directly detectable, for example, an antibody may be itself labeled with a fluorescent molecule. The binding partner also may be indirectly detectable, for example, a polynucleotide having a complementary nucleotide sequence can be a part of a branched DNA molecule that is in turn detectable through hybridization with other labeled polynucleotides. Quantitation of the signal is achieved
30 by, e.g., scintillation counting, densitometry, or flow cytometry.

"Linker" refers to a molecule that joins two other molecules, either covalently, or through ionic, van der Waals or hydrogen bonds, e.g., a polynucleotide

that hybridizes to one complementary sequence at the 5' end and to another complementary sequence at the 3' end, thus joining two non-complementary sequences.

"Amplification" refers to any means by which a polynucleotide sequence is copied and thus expanded into a larger number of polynucleotides, e.g., by reverse transcription, polymerase chain reaction, and ligase chain reaction.

"Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. Synthetic polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. The term "protein" typically refers to large polypeptides. The term "peptide" typically refers to short polypeptides.

Conventional notation is used herein to portray polypeptide sequences: the left-hand end of a polypeptide sequence is the amino-terminus; the right-hand end of a polypeptide sequence is the carboxyl-terminus.

"Antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically bind and recognize an analyte (antigen). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Antibodies exist, e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. This includes, e.g., Fab' and F(ab)'₂ fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized *de novo* using recombinant DNA methodologies.

An antibody "specifically binds to" or "is specifically immunoreactive with" a protein when the antibody functions in a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind preferentially to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions requires an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically

immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and Lane (1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

"Immunoassay" refers to an assay that utilizes an antibody to specifically bind an analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the analyte.

"Complementary" refers to the topological compatibility or matching together of interacting surfaces of two polynucleotides. Thus, the two molecules can be described as complementary, and furthermore, the contact surface characteristics are complementary to each other. A first polynucleotide is complementary to a second polynucleotide if the nucleotide sequence of the first polynucleotide is identical to the nucleotide sequence of the polynucleotide binding partner of the second polynucleotide. Thus, the polynucleotide whose sequence 5'-TATAC-3' is complementary to a polynucleotide whose sequence is 5'-GTATA-3'.

"Conservative substitution" refers to the substitution in a polypeptide of an amino acid with a functionally similar amino acid. The following six groups each contain amino acids that are conservative substitutions for one another:

- 1) Alanine (A), Serine (S), Threonine (T);
- 2) Aspartic acid (D), Glutamic acid (E);
- 3) Asparagine (N), Glutamine (Q);
- 4) Arginine (R), Lysine (K);
- 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
- 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

"Substantially pure" means an object species is the predominant species present (i.e., on a molar basis, more abundant than any other individual organic biomolecular species in the composition), and a substantially purified fraction is a composition wherein the object species comprises at least about 50% (on a molar basis) of all organic biomolecular species present. Generally, a substantially pure composition means that about 80% to 90% or more of the organic biomolecular species present in the composition is the purified species of interest. The object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional

detection methods) if the composition consists essentially of a single organic biomolecular species. "Organic biomolecule" refers to an organic molecule of biological origin, e.g., proteins, polynucleotides, carbohydrates or lipids. Solvent species, small molecules (< 500 Daltons), stabilizers (e.g., BSA), and elemental ion species are not
5 considered organic biomolecular species for purposes of this definition.

"Naturally-occurring" as applied to an object refers to the fact that the object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is
10 naturally-occurring.

"Pharmaceutical composition" refers to a composition suitable for pharmaceutical use in a mammal. A pharmaceutical composition comprises a pharmacologically effective amount of an active agent and a pharmaceutically acceptable carrier. "Pharmacologically effective amount" refers to that amount of an agent effective
15 to produce the intended pharmacological result. "Pharmaceutically acceptable carrier" refers to any of the standard pharmaceutical carriers, buffers, and excipients, such as a phosphate buffered saline solution, 5% aqueous solution of dextrose, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents and/or adjuvants. Suitable pharmaceutical carriers and formulations are described in
20 *Remington's Pharmaceutical Sciences*, 19th Ed. (Mack Publishing Co., Easton, 1995). Preferred pharmaceutical carriers depend upon the intended mode of administration of the active agent. Typical modes of administration include enteral (e.g., oral) or parenteral (e.g., subcutaneous, intramuscular, or intravenous intraperitoneal injection; or topical, transdermal, or transmucosal administration).

25 A population of cells is "senescent" if, after incubating a sub-confluent population of the cells with ^3H -thymidine for about 72 hours, the percentage of cells with labelled nuclei is below about 20%. A population of cells is "pre-senescent" if after the same treatment, at least 70% of the nuclei are stained. A cell is "senescent" if, after incubating the cell with ^3H -thymidine for about 72 hours, cell nucleus is not labelled or
30 is insignificantly labeled compared with cells of a pre-senescent cell population so treated.

Apart from these defining terms, senescent cell populations also can be identified by using bromodeoxyuridine, rather than ^3H -thymidine, as the label, and

detecting incorporation with anti-BrdU antibodies. Senescent cell populations also are characterized by an inability to undergo at least one population doubling when maintained in optimal growth media for four weeks.

5 A "senescent phenotype" or "senescent condition" is a phenotype or condition of senescent cells that differs from pre-senescent cells (e.g., increased collagenase expression), or a phenotype or condition of tissues, organs or organisms in which the action of senescent cells is a necessary component (e.g., traits of aged skin).

10 A "senescence responsive element" ("SnRE") is a nucleotide sequence identical or substantially identical to a nucleotide sequence located upstream of nucleotide -33 of the interstitial collagenase gene promoter that, in a senescent cell, up-regulates transcription of nucleotide sequences operatively linked to the promoter.

15 "Interstitial collagenase gene promoter" or "IC promoter" refers to the nucleotide sequence from nucleotide -1 to about nucleotide -500 of SEQ ID NO:2, as well as allelic forms thereof. A nucleotide sequence encoding the interstitial collagenase gene and 5' flanking sequence is further described in Burke et al., *supra*.

20 An element "up-regulates" transcription of a nucleotide sequence if transcription of a nucleotide sequence operatively linked to an expression control sequence including the element is greater than transcription of the nucleotide sequence operatively linked to the same expression control sequence excluding the element by a statistically significant amount.

25 "Minimal promoter" refers to expression control sequences that are necessary to engage the core polymerase, but not sufficient to drive transcription of a gene. The interstitial collagenase gene minimal promoter is located within nucleotides -1 to -34 of SEQ ID NO:2 and includes a TATA box. A minimal promoter is "heterologous" with respect to the senescence responsive element if it has no more than ten sequential nucleotides from nucleotides -1 to -34 of SEQ ID NO:2 between the initiation codon and the senescence responsive element. The CMV minimal promoter, the RSV minimal promoter, and the adenoviral late minimal promoter are examples of heterologous minimal promoters.

30 An expression control sequence is "essentially free of upstream sequences of the interstitial collagenase promoter" if sequences up to 500 nucleotides upstream of the senescence responsive element have less than 50% sequence identity over a window

of 20 nucleotides with sequences of the interstitial collagenase promoter upstream from nucleotide -134 of SEQ ID NO:1.

A nucleotide sequence is "heterologous" if it is not operatively linked with a senescence responsive element in nature. The interstitial collagenase gene is known to be operatively linked with the senescence responsive element in nature.

"Germ cell" refers to sperm cells or oocytes.

II. RECOMBINANT POLYNUCLEOTIDES COMPRISING THE SENESCENCE RESPONSIVE ELEMENT

This invention provides recombinant polynucleotides comprising an expression control sequence that includes (1) a senescence responsive element and (2) a minimal promoter positioned downstream of the senescence responsive element, in which the expression control sequence is operatively linked to a heterologous nucleotide sequence. The senescence responsive element is derived from the interstitial collagenase gene promoter. This promoter includes three transcriptional elements, PEA-3 (-89 to -82), CdxA (-81 to -75) and AP-1 (-72 to -66). Senescence responsive elements for use in the recombinant polynucleotides of the invention generally will include one or more of the elements PEA-3, CdxA and AP-1.

PEA-3 elements bind nuclear factors that share a common DNA-binding domain: the ets domain. Ets transcription factors comprise a family of related proteins, some of which have a helix-turn-helix domain (Wasylyk, B., *et al.* (1993) *Eur. J. Biochem.* 211:7-18). A tandem arrangement of ets and AP-1 sites has been documented in certain other promoters (Majerus, M.A., *et al.* (1992) *Nucleic Acids Res.* 20:2699-2703).

CdxA is a binding site for a homeobox transcription factor. Y. Margalite *et al.*, (1993) *Nucl. Res.* 21:4915-22. Its sequence is usually given as the complementary stand sequence to the sequence described here, i.e., 5'- cttata -3'.

AP-1 and ets transcription factors are known to interact to activate the promoters of a variety of genes. S.K. Logan *et al.* (1996) *J. Biol. Chem.* 271:774-782; A.G. Bassuk *et al.* (1995) *Immunity* 3:223-237; S. Bergelson *et al.* (1994) *Biochem. Biophys. Res. Commun.* 200:290-297; and A. Gutman *et al.* (1990) *EMBO Journal* 9:2241-2246.

While not wishing to be limited by theory, it is believed that an interaction between the PEA-3 and AP-1 elements, mediated by protein binding to the CdxA element is responsible for up-regulation by the senescence responsive element.

The SnRE used in the invention can comprise, for example, a sequence of
 5 at least 8 nucleotides, at least 15 nucleotides, at least 24 nucleotides, at least 30
 nucleotides; at least 50 nucleotides; at least 65 nucleotides; or 100 nucleotides
 substantially identical or identical to a nucleotide sequence from nucleotides -134 to -35
 of the IC gene promoter (from SEQ ID NO:1). In various embodiments, the SnRE can
 comprise any of the following sequences from the IC gene promoter (all from SEQ ID
 10 NO:1) as well as combinations (e.g., nucleotides -134 to -66) and fragments:

Nucleotides -89 to -66:

5'- gaggatgta taaagcatga gtca -3';

15 Nucleotides -95 to -60:

5'- aatcaa gaggatgta taaagcatga gtcagacacc -3';

Nucleotides -100 to -50:

5'- tagctaatcaa gaggatgta taaagcatga gtcagacacc tctggctttc -3';

20

Nucleotides -100 to -35:

5'- tagctaatcaa gaggatgta taaagcatga gtcagacacc tctggctttc tggaagggca aggac -3';

Nucleotides -134 to -35:

25 5'- agtc accatttcta atgattgcct agtctattca

tagctaatcaa gaggatgta taaagcatga gtcagacacc tctggctttc tggaagggca aggac -3'.

The recombinant polynucleotide generally will have the structure: 5' --
 senescence responsive element -- minimal promoter -- transcription initiation codon --
 30 sequence coding for expression of heterologous protein -- transcription termination codon
 -- transcription termination and polyadenylation sequence -- 3'. The recombinant
 polynucleotide is capable of increasing transcription of the nucleotide sequence in
 senescent cells and, in particular, fibroblasts. The sequence of the 5' upstream flanking

sequence is presented in Fig. 5 (SEQ ID NO:2), from Angel et al., *Molec. and Cell Biol.*, (1987) 7:2256-66. The entire coding sequence can be found in G.I. Goldberg et al., "Human fibroblast collagenase," *J. Biol. Chem.*, (1986) 261:6600-6605. The organization of the gene is described in Collier et al. "Structure of the human skin fibroblast collagenase gene," *J. Biol. Chem.* 263:10711-13 (1988).

Preferably, the senescence responsive element is placed just upstream of the minimal promoter. For example, the senescence responsive element can be attached essentially directly to the 5' end of the minimal promoter, no more than about 50 nucleotides upstream from the minimal promoter, or no more than about 100 nucleotides upstream from the minimal promoter.

In one embodiment, the recombinant polynucleotide utilizes sequences from the interstitial collagenase promoter. For example, the expression control sequences can comprise the interstitial collagenase minimal promoter, and, additionally, the senescence responsive element and sequences of several hundred nucleotides of the 5' flanking region, i.e., upstream from the initiation codon of the interstitial collagenase gene. This can include, for example, several thousand nucleotides of the 5' flanking region or fewer than about 500 nucleotides of the 5' flanking region.

In other embodiments, the expression control sequences comprise the senescence regulatory element and a heterologous minimal promoter, and/or is essentially free of upstream sequences of the interstitial collagenase promoter. The minimal promoter can be from any promoter known in the art including, for example, the adenoviral late promoter, the CMV promoter, the RSV promoter, the metallothionein promoter or the SV40 promoter.

The heterologous nucleotide sequence can code for the expression of a reporter polypeptide. Such constructs are useful to measure expression of a gene in a cell or to determine whether the cell is senescent. In this instance, the expression control sequence can comprise the interstitial collagenase promoter. Alternatively, the embodiment, the heterologous nucleotide sequence can code for the expression of polypeptides or functional polynucleotides whose expression one wishes to increase in senescent cells. For example, transcription regulators, interleukin-6, tissue inhibitors of metalloproteinases ("TIMPs"), collagens, elastin, growth regulatory transcription factors (e.g., E2F, c-fos, Id genes), and certain apoptosis regulators (e.g., *bax*) all are expressed in lowered quantities in senescent cells, in contrast to pre-senescent cells. The activities

of certain cyclin-dependent kinase activities also are lower in senescent cells.

Telomerase is not expressed in most somatic cells of most mammals. Therefore, the heterologous polynucleotide could code for expression of the RNA component or polypeptide components of telomerase. The RNA component of human telomerase is
5 described in J. Feng et al., "The RNA Component of Human Telomerase," (1995) *Science* 269:1236-1241 and Villeponteau et al., United States patent 5,583,016. Other molecules tend to be over-expressed in senescent cells. These include, for example, collagenase, metalloproteinases, plasminogen activators, inhibitors of plasminogen
10 activators, interleukin-1, insulin-like growth factors, inhibitors of cyclins or cyclin-dependent kinases. Accordingly, the heterologous nucleotide sequence can encode an inhibitor of these factors.

The polynucleotides used in the present invention can be cloned or amplified by *in vitro* methods, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), the transcription-based amplification system (TAS), the self-
15 sustained sequence replication system (3SR) and the Q β replicase amplification system (QB). For example, a polynucleotide can be isolated by polymerase chain reaction of human genomic DNA using primers based on known DNA sequences. A wide variety of cloning and *in vitro* amplification methodologies are well-known to persons of skill. PCR methods are described in, for example, U.S. Pat. No. 4,683,195; Mullis et al.
20 (1987) *Cold Spring Harbor Symp. Quant. Biol.* 51:263; and Erlich, ed., *PCR Technology*, (Stockton Press, NY, 1989). Polynucleotides also can be isolated by screening genomic libraries with probes selected from the sequences of SEQ ID NO:1 under stringent hybridization conditions, e.g., salt and temperature conditions substantially equivalent to 5x SSC and 65°C for both hybridization and wash.

25 Mutant versions of the senescence regulatory element can be made by site-specific mutagenesis or by random mutagenesis caused by increasing the error rate of PCR of the original polynucleotide with 0.1 mM MnCl₂ and unbalanced nucleotide concentrations.

This invention provides expression vectors, e.g., recombinant
30 polynucleotides further comprising expression control sequences operatively linked to the nucleotide sequence coding for expression of the polypeptide. Expression vectors are adapted for function in eukaryotes by inclusion of appropriate promoters, replication sequences, markers, etc. The construction of expression vectors and the expression of

genes in transfected cells involves the use of molecular cloning techniques also well known in the art. Sambrook et al., *Molecular Cloning -- A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, (1989) and *Current Protocols in Molecular Biology*, F.M. Ausubel et al., eds., (Current Protocols, a joint venture
5 between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (most recent Supplement).

Methods of transfecting genes into mammalian cells and obtaining their expression for *in vitro* use or for gene therapy, are well known to the art. See, e.g., *Methods in Enzymology*, vol. 185, Academic Press, Inc., San Diego, CA (D.V. Goeddel,
10 ed.) (1990) or M. Krieger, *Gene Transfer and Expression -- A Laboratory Manual*, Stockton Press, New York, NY, (1990).

Expression vectors useful in this invention depend on their intended use. Such expression vectors must, of course, contain expression and replication signals compatible with the host cell. Expression vectors useful in this invention include viral
15 vectors such as retroviruses, adenoviruses and adeno-associated viruses, plasmid vectors, cosmids, liposomes and the like. Viral and plasmid vectors are preferred for transfecting mammalian cells.

In another embodiment, endogenous genes are transcribed by operatively linking them to expression control sequences supplied endogenously that recombine with
20 genomic DNA. In one method, one provides the cell with a recombinant polynucleotide containing a targeting sequence, which permits homologous recombination into the genome upstream of the transcriptional start site of target gene; the expression control sequences; an exon of the target gene; and an unpaired splice-donor site which pairs with a splice acceptor in the target gene. Such methods are discussed in Treco et al., WO
25 94/12650; Treco et al., WO 95/31560 and Treco et al., WO 96/29411.

III. METHODS OF USING RECOMBINANT POLYNUCLEOTIDES COMPRISING THE SENESCENCE RESPONSIVE ELEMENT

Recombinant polynucleotides comprising the senescence responsive
30 element are useful for expressing the heterologous nucleotide sequences operatively linked to them in animal cells and, in particular, in mammalian cells. For model systems, the mammal can be a small mammal such as a rodent (e.g., a mouse) or a rabbit. In therapeutic methods, the mammal can be a human or any mammal treated in

veterinary medicine. More particularly, the recombinant polynucleotides are useful for obtaining increased expression of these nucleotide sequences in senescent cells. This expression is achieved by transfecting a cell with the recombinant polynucleotides of the invention. The expression control sequences are, of course, chosen to function in the recombinant host cell.

A. Isolated, Recombinant Host Cells

Accordingly, the invention also provides recombinant host cells comprising recombinant polynucleotides of this invention. The cells can be senescent cells, mixed passage cells, or pre-senescent cells. However, increased expression is expected in senescent cells. Many cell types can be transfected with these recombinant polynucleotides, including those cells known to express collagenase, especially fibroblasts (e.g., lung or skin), epithelial cells, stromal cells or any non-terminally differentiated cell.

Isolated recombinant host cells are useful *in vitro* to study the effect of increased gene expression on senescent cells. When the gene is a reporter gene, the cells are useful in screening methods to identify compounds that inhibit the activity of the senescence responsive element. They also are useful for administration into a subject for genetic therapy.

In animal subjects, *in vivo* transfection of cells with a recombinant polynucleotide of the invention is useful to restore levels of gene expression in senescent cells.

B. Transgenic Non-human Animals

Recombinant polynucleotides comprising the senescence responsive element also are useful for preparing transgenic animals whose senescent cells express the heterologous nucleotide sequence in greater amounts than in pre-senescent cells. Accordingly, this invention also provides transgenic non-human animals having a germ cell that comprises a recombinant polynucleotide of this invention. The animal can be a mammal. Such animals are useful in the study of senescence. For example, the heterologous nucleotide sequence can encode a reporter gene. Cells of the animal can be examined at various points in its life to determine whether the cells are senescent or not. Cell from various organs can be tested to determine whether the organs senesce at the

same rate. Compounds can be administered to the animal to determine what effect they have on senescence.

In one embodiment, the non-human mammal further comprises germ cells comprising a recombinant polynucleotide comprising second expression control sequences operatively linked to a second nucleotide sequence coding for expression of, for example, a collagenase inhibitor, a p21 inhibitor, a cyclin dependent kinase, a p53 tumor suppressor or a retinoblastoma tumor suppressor.

The transgenic animals of this invention are usually produced by introducing the recombinant polynucleotide into a fertilized oocyte or embryonic stem (ES) cell, typically by microinjection, electroporation, lipofection, particle-mediated gene transfer. The transgenic animals express the heterologous nucleotide sequence in tissues depending upon whether the promoter is inducible by a signal to the cell, or is constitutive. Transgenic animals can be bred with non-transgenic animals to produce transgenic animals with mixed characteristics.

Using standard methods, transgenic mice have been produced that include recombinant polynucleotides having either nucleotides -34 to +34 of the IC gene promoter operatively linked with a β -galactosidase reporter gene, or -134 to +34 of the IC gene promoter operatively linked with a β -galactosidase reporter gene. The transgenic mice integrated into their genome the construct containing the expression control sequences including a senescence responsive element.

C. Expression Assays

Recombinant polynucleotides comprising the senescence responsive element also are useful in a variety of assays involving detection of the expression of reporter genes in senescent cells. Such assays are useful to determine whether a cell is senescent, because senescent cells will exhibit higher expression of such reporter genes than pre-senescent cells.

In one aspect, this invention provides methods of identifying senescent cells in a population or in an organism involving providing recombinant host cells harboring a recombinant polynucleotide of the invention that comprises the senescence responsive element operatively linked with a reporter gene, or providing a transgenic non-human animal whose cells comprise a reporter gene operatively linked with the senescence responsive element. Then, the amount of expression of the reporter gene is

determined under selected conditions. This amount is compared with a control amount that reflects the expected amount of expression in a pre-senescent cell. Thus; the amount measured in the test cell reflects whether the cell is senescent or not. The assay can be run on populations of cells to determine the percent of senescent cells in the population.

- 5 The cells can be isolated, recombinant cells *in vitro*, cells transfected *ex vivo* and introduced into the animal, or cells of a transgenic animal. Thus, the vector is introduced into the cells to be tested.

D. Screening Assays

- 10 In another aspect, this invention provides screening methods to identify compounds that alter the activity of the senescence responsive element. The methods involve providing a recombinant host cell transfected with a recombinant polynucleotide comprising an expression control sequence operatively linked to a reporter gene, wherein the expression control sequence comprises a senescence responsive element located
- 15 upstream of a minimal promoter; or providing a transgenic non-human animal whose cells comprise a reporter gene operatively linked with the senescence responsive element. Then, the cell or animal is exposed to a test compound. The amount of expression of the reporter gene is determined and compared with a control amount. If the amount of expression is different than the control amount, then the compound alters the activity of
- 20 the senescence responsive element.

- Frequently, the reporter will be an enzyme. Methods of assaying for reporters are well known in the art and generally include exposing a sample to a substrate for the enzyme and determining the amount or rate at which the enzyme catalyzes the substrate. The amount of expression in single cells can be measured if the
- 25 signal provided by the reporter is visible, such as a fluorescent signal. For example, if the heterologous nucleotide sequence encodes a fluorescent protein, such as green fluorescent protein, fluorescence in a single cell can be detected and measured using, for example, a fluorescent cell sorter. See, e.g., Heim et al., WO 96/23810.

30 IV. POLYNUCLEOTIDE PROBES

This invention is also directed to polynucleotide probes, preferably isolated, that specifically hybridize with a nucleotide sequence of SEQ ID NO:1 or its complement. The probes typically have at least 15 nucleotides, at least 20 nucleotides or

at least 25 nucleotides. The probes can have a sequence identical to or complementary to the sequence of SEQ ID NO:1. In one embodiment, the probes are no more than about 50 nucleotides in length. In one embodiment, the isolated polynucleotides further comprise a label. These isolated polynucleotides are useful as primers for amplification of senescence responsive element sequences by, e.g., PCR. They also are useful as probes in hybridization assays, such as Southern and Northern blots, for identifying polynucleotides having a nucleotide sequence of a protein of this invention. Probes of this invention that hybridize with the senescence responsive element are useful as inhibitory polynucleotides to suppress expression of nucleotide sequences operatively linked with the senescence responsive element. Polynucleotide probes can be introduced into a cell by any of the means already described. Polynucleotide probes can be made by any means known to the art including, for example, chemical synthesis, PCR and expression from expression vectors.

V. CONSTRUCTS AND METHODS FOR INHIBITING THE ACTIVITY OF THE SENESCENCE RESPONSIVE ELEMENT

A. Inhibitory Polynucleotides

Nucleotide sequences operatively linked to the senescence responsive element are over-expressed in senescent cells. This invention provides constructs and methods for inhibiting the expression of such nucleotide sequences using inhibitory polynucleotides. Inhibitory polynucleotides are single-stranded polynucleotides that can inhibit the function of a target nucleotide sequence. Without wishing to be limited by theory, inhibitory polynucleotides may function by hybridizing to the polynucleotide they inhibit. Typically, an inhibitory polynucleotide has at least about 55% sequence identity over a stretch of at least 6-25 nucleotides (e.g., 6, 10, 15, 20 or 25), preferably at least about 65%, more preferably at least about 75%, and most preferably at least about 90%. See M. Kanehisa, *Nucleic Acids Res.* (1984) 12:203.

By binding to the appropriate target sequence, a DNA-DNA, DNA-RNA or RNA-DNA duplex is formed. Certain of these polynucleotides are termed "antisense" because they are usually complementary to the sense, or coding, strand of the gene. Recently, approaches for use of "sense" inhibitory polynucleotides have also been developed. The term "inhibitory polynucleotides" as used herein, refers to both "sense" and "antisense" polynucleotides.

By binding to the target polynucleotide, the inhibitory polynucleotide can inhibit the function of the target polynucleotide. This could, for example, be a result of blocking DNA replication or DNA transcription, e.g., by interfering with the attachment of the polymerase to the promoter; interfering with processing of, poly(A) addition to, or translation of mRNA; or promoting inhibitory mechanisms of the cells, such as promoting RNA degradation. Inhibitory polynucleotide methods therefore encompass a number of different approaches to altering expression of specific genes that operate by different mechanisms. These different types of inhibitory polynucleotide technology are described in C. Helene and J. Toulme, (1990) *Biochim. Biophys. Acta.*, 1049:99-125.

Inhibitory polynucleotide approaches can be classified into those that target DNA sequences, those that target RNA sequences (including pre-mRNA and mRNA), those that target proteins (sense strand approaches), and those that cause cleavage or chemical modification of the target polynucleotides.

Approaches targeting DNA fall into several categories. Polynucleotides can be designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory polynucleotides are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. Helene and Toulme, *supra*.

The inhibitory polynucleotides can also be targeted against mRNA. In this approach, the inhibitory polynucleotides are designed to hybridize to a target sequence in the mRNA and, thereby, interfere with its translation. Translation of mRNA is inhibited if there is a measurable decrease in the amount of protein produced by the cell after treatment. Inhibiting translation of an mRNA results in suppression of the cellular function carried out by the encoded protein. For example, an inhibitory polynucleotide complementary to regions of c-myc mRNA inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which over-expresses the c-myc proto-oncogene. See E.L. Wickstrom et al., (1988) *PNAS (USA)*, 85:1028-1032 and Harel-Bellan et al., (1988) *Exp. Med.*, 168:2309-2318. Maier et al., (1990) *Science*, 249:1570-74 showed how to suppress interleukin-1 α activity using unmodified antisense oligonucleotides. As described in Helene and Toulme, *supra*, inhibitory polynucleotides targeting mRNA have been shown to work by several different mechanisms in order to inhibit translation of the encoded protein(s).

Whatever the ultimate strategy, it is desirable to provide oligomers with physiological properties which render them more effective. The general approach to constructing various polynucleotides useful in inhibitory polynucleotide therapy has been reviewed by A.R. Vander Krol *et al.* (1988), *Biotechniques* 6:958-976, and by C.A.

- 5 Stein *et al.*, (1988) *Cancer Res.* (1988) 48:2659-2668. See also *Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression*, Cohen, J.S., editor, MacMillan Press, London, pages 79-196 (1989), and *Antisense RNA and DNA*, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

10 B. Delivery Of Inhibitory Polynucleotides

This invention contemplates a variety of means for delivering the inhibitory polynucleotide to the cell including, for example, direct uptake of the molecule by the cell from solution, facilitated uptake through liposome vectors and intracellular expression from an expression cassette.

- 15 One can provide a cell with an inhibitory polynucleotide by contacting the cell with a soluble inhibitory polynucleotide, for example, in the culture medium *in vitro* or in the circulatory system, interstitial fluid or tissue mass *in vivo*. Soluble inhibitory polynucleotides present in the external milieu have been shown to gain access to the cytoplasm.

- 20 One also can provide inhibitory polynucleotides by expressing them inside a cell using expression vectors having an expression control sequence operatively linked to a nucleotide sequence that encodes the inhibitory polynucleotide. The nucleotide sequence hybridizes with a sequence identical to or complementary to at least 6 nucleotides within nucleotides -35 to -134 of SEQ ID NO:1. The sequence can be no
25 more than 30 nucleotides.

VI. PROPHYLACTIC AND THERAPEUTIC TREATMENTS

A. Treating Senescent Phenotypes and Conditions

- 30 The activity of senescent cells differs from pre-senescent cells and results in certain of the senescent phenotypes or conditions characteristic of aged cells, tissues, organs and organisms. Certain of the senescent phenotypes result from over-expression of genes under the transcriptional control of senescence responsive elements, while others result from under-expression of genes. Accordingly, these phenotypes can be prevented,

diminished or reversed by decreasing expression of over-expressed genes by interfering with the activity of genes already under the transcriptional control of a senescence responsive element, or by increasing expression of under-expressed genes by putting them under the control of a senescence responsive element. The phenotypes can be treated in aged subjects, subjects suffering from a pro-geriatric disease, such as Werner's syndrome or Hutchinson-Guilford syndrome, among others, or in non-aged subjects for prophylaxis.

Genes over-expressed in senescent cells, such as the interstitial collagenase gene, are under the control of a senescence responsive element. Two senescent phenotypes associated with over-expression of interstitial collagenase are aged stroma underlying epithelial organs and epithelial cancers. Epithelial organs include, for example, skin, lung, stomach, intestine, pancreas, kidney, blood vessels, and liver. Aged epithelial organs are characterized by a loss in function and in integrity of the stroma and epithelium. For example, skin exhibits wrinkling, loss of elasticity, and dryness resulting at least in part from breakdown of collagen.

Increased risk of cancer also is a senescent phenotype. Senescent fibroblasts secrete high levels of collagenase and stromelysin, which tend to destroy the dermal matrix. This destruction alters the micro-environment of epithelial cells. Senescent fibroblasts also secrete high levels of heregulin, a ligand for an erbB receptor. Heregulin stimulates the growth of epithelial tumors that have an amplification of the erbB2 gene. It is believed that as a result of the destruction of the dermal matrix, these initiated epithelial cells can become cancerous, separate from the epithelium and metastasize. Thus, an age-dependent accumulation of senescent cells in mitotic tissues creates a pro-carcinogenic environment.

More generally, it is believed that other senescent phenotypes result from over-expression of genes under the control of a senescence responsive element. For example, the aged tend to have a general increase in inflammation. This appears to result at least in part from the over-expression of inflammatory mediators. Diseases of the aged that include an inflammatory component include, for example, osteoarthritis (inflammation of the joints), Alzheimer's disease (inflammation of the brain) and atherosclerosis (inflammation of the blood vessels).

Accordingly, treatments involve targeting cells of the subject that over-express the gene which is a factor in the phenotype with an agent that inhibits expression

of genes operatively linked to the IC gene promoter. More particularly, the agent can interfere with the activity of the senescence responsive element. For example, in the treatment of aging skin or prophylaxis of cancer, epithelial cells, and, in particular, fibroblasts, can be targeted.

5 Certain genes are under-expressed in senescent cells. Up-regulating these genes is useful in therapies to restore function diminished from under-expression. For example, IL-6 is under-expressed in senescent cells. IL-6 is produced by monocytes, macrophages, T_H2 cells and bone marrow stromal cells. It promotes differentiation of into plasma cells and stimulates antibody secretion. Therefore, up-regulating IL-6
10 production in senescent cells is useful in restoring certain immune system functions.

B. Therapy With Polynucleotides

 The recombinant polynucleotides of this invention are useful in gene therapy. In one aspect, recombinant polynucleotides that have expression control
15 sequences comprising the senescence responsive element are useful for expressing genes in senescent cells. In particular, such expression vectors are useful for expressing genes that are under-expressed or that are not expressed in senescent cells, or simply for expressing genes at high levels in these cells.

 In another aspect, one can deliver inhibitory polynucleotides or
20 recombinant polynucleotides that code for the expression of inhibitory polynucleotides to a cell.

 Host cells can be transfected by any methods known to the art. For example, methods for retroviral transfection are described in, for example, M. Krieger (33) and *Methods in Enzymology*, Vol. 185 (34). Cells can be transfected with plasmid
25 vectors, for example, by electroporation. Cells can be transfected by recombinant polynucleotides by calcium phosphate precipitation DNA liposome complexes or by particle-mediated gene transfer (biolistics). Methods are also known for transfection of polynucleotides using liposomes.

30 C. Pharmaceutical Compositions

 The therapeutic polynucleotides can be delivered by any route and in any suitable vehicle. The agent of the invention molecule can be administered to the subject in a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a

pharmacologically effective amount the agent of the invention. A pharmacologically effective amount of the agent of the invention depends upon the intended result, e.g., expression of a nucleotide sequence from the senescence responsive element or inhibition of the senescence responsive element with an inhibitory polynucleotide.

5 The pharmaceutical compositions are intended for all well known forms of administration and, in particular, parenteral administration for prophylactic and/or therapeutic treatment. Local administration, such as transdermally, also is contemplated. The pharmaceutical compositions can be administered in a variety of unit dosage forms depending upon the method of administration. For example, unit dosage forms for
10 parenteral administration include unit doses of injectable solutions.

 The agent of the invention can be administered by any means known in the art for delivery of proteins. However, systemic administration by injection is preferred. This includes intramuscular, intravenous, intraperitoneal, and subcutaneous injection.

 The form, amounts and timing of administration generally are a matter for
15 determination by the practitioner. In one embodiment, the pharmaceutical composition is delivered as a unit dosage form. Dosages include about of 10^7 to 10^{13} particles of viral vector per ml of carrier. The volume administered can be selected by the practitioner. According to one embodiment of this invention, approximately 10^{10} vectors suspended in about 1 ml of sterile PBS constitute a therapeutically effective amount.

20

VII. ISOLATING MOLECULES THAT BIND TO THE SENESCENCE RESPONSIVE ELEMENT

 Polynucleotides comprising sequences for the senescence responsive element are useful in screening methods to identify molecules that bind to the element
25 and through which it may function. One such method involves screening a phage-based human fibroblast cDNA library with a labeled polynucleotide probe of the invention. In another method a yeast strain is created containing recombinant polynucleotides comprising a recombinant polynucleotide having expression control sequences comprising tandem senescence responsive elements and a minimal promoter operatively linked to a
30 heterologous nucleotide sequence encoding a selection or reporter gene. A human cDNA library fused to a yeast transcription activator domain is then used to select human cDNAs that bind the senescence responsive element. In another method, such molecules can be isolated by creating affinity matrices to which the probe is attached. The test

sample is exposed to the matrix, and molecules that bind the senescence responsive element are isolated.

The following examples are offered by way of illustration, not by way of limitation.

5

EXAMPLES

I. MAPPING THE SENESCENCE RESPONSIVE ELEMENT

A. Materials and Methods

WI-38 human fetal lung fibroblasts were used at 60-80% confluence. For
10 all experiments, the growth potential of each culture was determined by labeling sub-confluent cells with ³H-thymidine for 72 h and counting the fraction of cells with labeled nuclei (LN) after autoradiography. In general, senescent cells had less than 10% LN, whereas pre-senescent cells had greater than 70% LN. The data shown are typically the average of three to five independent experiments, with each data point being the average
15 of duplicates.

A Pst I restriction fragment from the plasmid pCLCAT3 (Frisch et al., *Oncogene*, 1990, 5:75) contained 3.4 kb of the 5' flanking sequence of the human interstitial collagenase gene. This fragment encompassed position +37 to position -3470, where position +1 is the start of transcription. The fragment was inserted into the Pst I
20 site in the multiple cloning site of the vector pBluescript II KS (+) from Stratagene (La Jolla, CA). This generated the plasmid pIC-1.

Restriction of pIC-1 with Kpn I and Bam HI yielded a fragment containing the promoter region up to position -521. This fragment was cloned into the luciferase reporter vector pGL3-basic (Promega Corp., Madison, WI) between the Ipn I and Bgl II
25 sites. This construct was named pGL-522 because it contained the 5' flanking region of the human interstitial collagenase from positions +37 to position -522 (having reconstituted the Kpn I site). pGL-522 served as the basis for all the other reporter constructs, so that all constructs shared a common 3' end of the collagenase promoter.

pGL-3470 was constructed by cutting pIC-1 with Kpn I, recovering the
30 3 kb fragment, and cloning it into the Kpn I site of pGL-522. Restriction of pGL-3470 with Eco RI, recovery of the largest fragment and self-ligation of this fragment produced the construct pGL-3030. Restriction of pGL-3470 with Eco RV and Pvu II, recovery of the largest fragment and self-ligation of this fragment produced pCL-2490. Digestion

of pGL-3470 with Eco RI and Bgl II, end-filling, recovery of the largest fragment and self-ligation generated pGL-1210.

Pfu DNA polymerase was used in the polymerase chain reaction to amplify from pGL-522 three DNA fragments using the following primers:

- 5 1) 5' ccaaataatc tgctagcagt caccatttct aatgattgcc (SEQ ID NO:3)
- 2) 5' ggctttctgg aagggaacg cgctatata tacagaggga gc (SEQ ID NO:4)
- 3) 5' gttttggcg tcttccatgg tggtttacc (SEQ ID NO:5)
- 4) 5' gcgcagtgtt aggaattcca ccctctgcc (SEQ ID NO:6)

Primers 1 and 3 were used to generate an Nhe I-Nco I fragment, primers 2 and 3 were used to generate an Mlu I-Nco I fragment, and primers 3 and 4 were used to generate an Eco RI-Nco I fragment. After digesting the amplified fragments with the appropriate restriction endonucleases, they were cloned into pGL3-basic (cut with Nhe I/Nco I or Mlu I/Nco I) or PGL-3400 (cut with Eco RI and Nco I; the largest restriction fragment was recovered). This gave rise to the constructs pGL-134 (containing nucleotides -134 to +37), pGL-34 (containing nucleotides -34 to +37) and PGL-390 (containing nucleotides -390 to +37), respectively. The construct PGL-522 Δ 34/134 contained the collagenase promoter up to position -522 but with the region between positions -34 and -134 deleted. This was constructed by amplifying a fragment from PGL-522 using the following primers:

- 20 5) 5' aatagggtac caggcagctt aacaaaggc (SEQ ID NO:7)
- 6) 5' agaaatggtg acgcgtagca gattatttg aatcactt (SEQ ID NO:8)

This fragment, containing the Kpn I and Mlu I sites, was cloned into PGL-34 cut with the same enzymes.

Cells were transfected by electroporation using Cytomix buffer (van den Hoff et al., 1992, Nucl Acids Res 20:2902). The composition of the buffer is as follows: 120 mM KCl, 0.15 mM CaCl₂, 10 mM K₂HPO₄, 10 mM KH₂PO₄, 25 mM HEPES, 2 mM EGTA, 5 mM MgCl₂ (pH adjusted to 7.6 with KOH). Cytomix was made fresh or stored frozen, to avoid precipitation of the salts. Immediately prior to electroporation, 2 mM ATP and 5 mM glutathione was added and the pH was readjusted to 7.6.

WI-38 cells were trypsinized, washed with DME medium and spun at low speed in a bench-top centrifuge. The cell pellets were washed with 10 ml of fresh cytomix buffer and counted with a Coulter counter. 1×10^6 senescent cells were used

for each electroporation. Pre-senescent cells were used at 3×10^5 up to 1×10^6 . The cells were spun again at low speed, and then resuspended in enough fresh cytomix buffer (containing ATP and glutathione) to provide 400-500 μ l cell suspension per cuvette (Biorad 0.4 cm electrode gap).

5 Plasmid DNA was added to the cuvettes, and the suspension was mixed gently and incubated on ice for at least 5 min. Using the luciferase PGL 3 reporter system (Promega Corp., Madison, WI), 1 μ g of the reporter plasmid easily yielded measurable activity of the collagenase gene promoter in senescent cells. 0.5-1 μ g of a β -galactosidase reporter gene driven by the human CMV promoter (CMV- β GAL) was
10 included to control for transfection efficiency. CMV- β GAL yielded readily measurable activity using a chemiluminescent β -galactosidase assay (Clontech, Palo Alto, CA).

Senescent cells were electroporated at 240-270 V and 950 μ F using a Biorad Gene Pulser II apparatus. Pre-senescent cells were electroporated at 300 V, 950 μ F. Before delivering the pulse, the sample resistance was measured to confirm that
15 the resistance was 20 ohms.

After the pulse was delivered, the cuvettes were placed on ice. As soon as all cuvettes were pulsed, the electroporated cells were seeded in 35 mm dishes containing DME medium plus 10% fetal calf serum and incubated at 37°C for 1 h. Because most of the electroporated cells formed floating clumps, at 1 h intervals, a sterile pipette was
20 used to gently pipet the medium, being careful not to dislodge cells that had already attached, in order to break up the cell clumps. This operation was repeated three or four times until most cells attached to the dish. After 6-12 h the medium was replaced with fresh DME containing 10% fetal calf serum.

The transfected cells were cultured for 72 h from the start of
25 electroporation, then lysed to extract protein and assay luciferase and β -galactosidase activity. Luciferase was from assayed using a kit from Promega (Madison, WI), and the reporter Lysis Buffer to prevent inhibition of β -galactosidase activity. β -galactosidase activity was determined using a Luminescent β -galactosidase assay system from Clontech (Palo Alto, CA). The luciferase activity was divided by the β -galactosidase activity in
30 each sample to calculate a normalized luciferase activity. The normalized luciferase activity reflects the transcriptional activity of the collagenase promoter fragments, normalized for any cuvette-to-curette variation in transfection efficiency or toxicity due to the electroporation.

B. Localization of Senescence Responsive Element Activity to Nucleotides -34 to -134

Reporter constructs PGL-34, PGL-134, PGL-390, PGL-522, PGL-522 delta 34/134 and PGL-1210 were tested for activity in senescent cells. Figure 3 shows the results. Most of the sequence responsible for transcription in senescent cells mapped to nucleotides -34 to -134. More particularly, PGL-134 showed a 20.8-fold increase in transcription compared with the minimal promoter, PGL-34. Adding the nucleotides -135 to -522 increased transcription about another 4-fold. However, removal of nucleotides -34 to -134 from PGL-522 (PGL-522 delta 34/134) reduced transcription to only about 3-fold over the minimal promoter.

C. Localization Of Senescence Responsive Element Activity To Nucleotides -34 to -100

Reporter constructs linking short fragments of the collagenase promoter to a luciferase gene were transfected into pre-senescent and senescent human fibroblasts (WI-38 cells). These mapping experiments localized the SnRE to a 66 bp region between positions -34 and -100.

TABLE I

Construct	Fold activity over minimal promoter
PGL-34	1 +/-0.5
PGL-77	4.1 +/-1.7
PGL-100	27.5 +/-6
PGL-134	29.6 +/-8

D. Role Of PEA-3 And AP-1 Elements In Senescence Responsive Element Activity

A construct lacking the PEA-3 site, but containing the AP-1 site (i.e., terminating at position -77), failed to drive reporter activity in senescent cells. By contrast, the construct extending up to position -100, which included both the PEA-3 and AP-1 sites, exhibited 93% of the activity of the largest construct tested in this experiment (up to position -134). This result indicates that sequences around the PEA-3 site are necessary for SnRE activity.

Two additional luciferase reporter constructs, one carrying a mutated PEA-3 site and a wild type (wt) AP-1 site and a second carrying a mutated AP-1 site flanked by a wt PEA-3 site were transfected into senescent WI-38 fibroblasts. The sequences of the wild type and the mutant sites are given below:

5	wt PEA-3 + wt AP-1:					
		-89	-82	-72	-66	
		<u>agaggatggt</u>	<u>ataaagcatg</u>	<u>agtca</u>		(SEQ ID NO:9)
		PEA-3	CdxA	AP-1		
10	mutant PEA-3 + wt AP-1:					
		<u>agagtacttt</u>	<u>ataaagcatg</u>	<u>agtca</u>		(SEQ ID NO:10)
		* **				
15	wt PEA-3 + wt AP-1:					
		<u>agaggatggt</u>	<u>ataaagcatg</u>	<u>agtcagac</u>		(SEQ ID NO: 11)
		PEA-3	CdxA	AP-1		
20	wt PEA-3 + mutant AP-1					
		<u>agaggatggt</u>	<u>ataaagcagg</u>	<u>attcctac</u>		(SEQ ID NO: 12)
			* *	**		

Mutations in the AP-1 site completely abolished reporter activity. This result indicates that the AP-1 site is necessary for SnRE activity.

25 Mutations in the PEA-3 site reduced promoter activity, albeit not as strongly as mutations in the AP-1 site, resulting in a 40% decrease in activity. This results suggests that the PEA-3 site at least partially contributes to SnRE activity. Combined with the deletion experiment described above, the results further suggest that the CdxA site also is involved in SnRE activity. The data indicate that the PEA-3, CdxA
30 and AP-1 sites are required for optimal activity of the collagenase promoter in senescent cells.

TABLE II

	<u>Construct</u>	<u>Fold activity over minimal promoter</u>
35	PGL-34	1.0 +/-0.6
	PGL-100 (wt PEA3/wt AP1)	26.0 +/-6.7
	PGL-100 (wt PEA3/mut AP1)	1.3 +/-0.4
	PGL-100 (mut PEA3/wt AP1)	16.0 +/-5

II. SENESCENCE RESPONSIVE ELEMENT ACTIVITY IN PRE-SENESCENT AND SENESCENT CELLS

A. Active versus Quiescent Cells

Previous experiments showed that SnRE was active in senescent cells maintained in 10% serum, but not in pre-senescent cells growing in 10% serum. It was possible that differences in SnRE activity between pre-senescent and senescent cells reflected differences in their growth states, rather than differences in their replicative potentials. Pre-senescent WI-38 fibroblasts were made quiescent by depriving them of serum for 4 days, and used to determine whether the SnRE was also active in quiescent cells. These experiments showed that activity of the collagenase promoter up to position -522 was not significantly higher in quiescent cells, compared to actively growing cells.

TABLE III

Construct	<u>Fold activity over minimal promoter</u>	
	<u>Growing Cells</u>	<u>Quiescent Cells</u>
PGL-34	1.0 +/-0.2	1.0 +/-0
PGL-134	2.3 +/-0.7	2.7 +/-0.85
PGL-522	2.6 +/-0.7	3.6 +/-1.5

B. Senescent versus Pre-senescent Cells

Bacterial β -galactosidase (β gal) reporter vectors were constructed in order to determine SnRE activity in individual cells.

The CMV promoter was excised from pCMV β (Clontech) and was either recircularized (generating a promoterless plasmid) or ligated to the -34/+37 or -134/+37 fragments from the human interstitial collagenase promoter. The constructs thus obtained were called PO (promoterless), P-34 (collagenase promoter lacking the SnRE) and P-134 (collagenase promoter containing the SnRE), respectively. PO and P-34 served as controls to rule out the existence of cryptic senescence activated promoters or enhancers in the vector.

Heterogeneous cultures of primary mouse embryo fibroblasts and human fibroblasts were transfected. Mouse cultures contained roughly 50% pre-senescent/50% senescent cells, whereas the human cultures contained 25% pre-senescent/75% senescent cells. After transfection, the cells were cultured for 72 h in medium containing tritiated thymidine. The cells were then fixed and incubated with the β gal substrate X-gal, which

stains β gal-expressing cells blue. After staining, the cells were subjected to autoradiography, which identifies cells that underwent DNA synthesis during the labeling period (incorporated radiolabel into nuclei).

Experiments show that only pre-senescent cells show nuclear radiolabeling; senescent cells do not synthesize DNA and therefore do not label with tritiated thymidine. If the SnRE is active preferentially in senescent cells, the majority of reporter-positive (blue) cells should be devoid of radiolabel. Thus, this experiment distinguishes senescent from pre-senescent cells by nuclear labeling, and, in the same cells, determines whether or not the SnRE is active. The pooled results of several such experiments are shown below.

TABLE IV

Mouse Fibroblasts: Ave. labeling index = 56.5% (53%-60%)

	Construct	Number of Blue Cells Detected	% Labeled Nuclei	Blue Cells with Labeled Nuclei	Blue Cells, % Labeled Nuclei
15	None	0	56.5	Not Applicable	Not Applicable
	P0	1		0	Not Significant
	P-34	4		1	Not Significant
	P-134	669		64	9.6

TABLE V

Human Fibroblasts: Ave. labeling index = 23.5% (14.8-34%)

	Construct	Number of Blue Cells Detected	% Labeled Nuclei	Blue Cells with Labeled Nuclei	Blue Cells, % Labeled Nuclei
25	None	0	23.5	Not Applicable	Not Applicable
	P0	1		0	Not Significant
	P-34	5		1	Not Significant
	P0134	338		25	7.4

P0 (promoterless vector) and P-34 (vector containing the collagenase minimal promoter only) were essentially incapable of driving β gal reporter expression. Cells expressing reporter activity (blue cells) were abundant only in cultures transfected with vector in which the SnRE was present upstream of the collagenase minimal promoter.

Most cells in which the SnRE was active (blue cells) did not incorporate tritiated thymidine (< 10% labeled nuclei). By contrast, the bulk of the cell population had a much higher labeling index (53-60% for mouse cultures, and 14-34% for human cultures). Because cells were incubated with radiolabel for 3 days prior to staining for β gal (SnRE) activity, this experiment overestimates the number of radiolabeled cells showing SnRE activity. Thus, the SnRE was preferentially active in (unlabeled) senescent fibroblasts, and this was true of both mouse and human fibroblasts.

β -galactosidase activity itself was not responsible for the lack of DNA synthesis β gal-positive cells. Pre-senescent human fibroblasts expressing pCMV β gal (β -galactosidase under control of the CMV promoter) synthesized DNA to similar extends as untransfected cells or cells transfected with a control vector.

Most of the cells with an active SnRE (blue cells) had a large, flattened morphology characteristic of senescent cells.

C. Activity Of Stably Integrated Senescence Responsive Element

The experiments described above utilized transiently transfected plasmids, the majority of which do not integrate into the genome. In this experiment, PGL3 or PGL-134 and a plasmid carrying a neomycin resistance gene were co-transfected into HCA-2 human neonatal foreskin fibroblasts. PGL3 lacks a promoter upstream of the luciferase reporter gene. PGL-134, by contrast, contains the region +37 to -134 of the collagenase promoter, which contains the SnRE, upstream of the reporter gene. Stable transfectants were selected by growing the cells in a neomycin-related drug (G-418). Only one stably transfected clone from each transfection had sufficient replicative potential to test reporter (luciferase) activity as the cells approached senescence. Cells were monitored for replicative potential, as judged by the percent nuclei that incorporated tritiated thymidine in 72 hours (% Labeled Nuclei), and luciferase activity (RLU, relative luminescence units). As the cells lost replicative potential, SnRE (luciferase) activity increased.

TABLE VI

	PGL3			PGL-134		
	% Labeled Nuclei	RLU/ mg. protein	Fold Increase	% Labeled Nuclei	RLU/ mg. protein	Fold Increase
5	33	683	1.0	55	1162	1.0
	27	310	0.5	38	3175	2.7
	5	205	0.3	14	10138	8.7

10 III. THE SNRE CONFERS SENESCENCE-DEPENDENT RESPONSIVENESS TO A HETEROLOGOUS PROMOTER

Experiments demonstrate that SnRE confers senescence-dependent regulation on a heterologous promoter. Plasmid pAD β (Clontech, Palo Alto, CA) contains the minimal promoter from the major late genes of adenovirus 2 linked to a β gal reporter gene. Nucleotides -34 to -134 of the interstitial collagenase gene promoter was cloned upstream of the minimal promoter in pAD β to generate pAD β SNRE. Primary mouse embryo fibroblasts were transfected with pAD β and pAD β SNRE, labeled for 72 h with tritiated thymidine, and assayed for labeled nuclei and β gal activity. The results of several experiments are shown below.

TABLE VII

Mouse Fibroblasts: Ave. labeling index = 65.1%

	Construct	Number of Blue Cells Detected	% Labeled Nuclei	Blue Cells with Labeled Nuclei	Blue Cells, % Labeled Nuclei
25	None	0	65.1	Not Applicable	Not Applicable
	pAD β	5		0	Not Significant
30	pAD β SNRE	1011		182	18.0

The present invention provides a novel transcriptional regulator in senescent cells methods of using it. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above

description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each
5 individual publication or patent document were so individually denoted.

WHAT IS CLAIMED IS:

- 1 1. A recombinant polynucleotide comprising an expression control
2 sequence operatively linked to a heterologous nucleotide sequence, the expression control
3 sequence comprising (1) a senescence responsive element and (2) a heterologous minimal
4 promoter positioned downstream of the senescence responsive element, wherein the
5 senescence responsive element up-regulates transcription of the heterologous nucleotide
6 sequence in a senescent cell.
- 1 2. The recombinant polynucleotide of claim 1 wherein the senescence
2 responsive element comprises nucleotides -89 to -66 of the interstitial collagenase gene
3 promoter (5'- gaggatgtta taaagcatga gtca -3' (SEQ ID: NO:1)).
- 1 3. The recombinant polynucleotide of claim 1 wherein the senescence
2 responsive element comprises nucleotides -100 to -50 of the interstitial collagenase gene
3 promoter (5'- tagctaatacaa gaggatgtta taaagcatga gtcagacacc tctggctttc -3) (SEQ ID:
4 NO:1)).
- 1 4. The recombinant polynucleotide of claim 1 wherein the senescence
2 responsive element comprises nucleotides -134 to -35 of the interstitial collagenase gene
3 promoter (5'- agtc accatttcta atgattgcct agtctattca tagctaatacaa gaggatgtta taaagcatga
4 gtcagacacc tctggctttc tggaagggca aggac -3') (SEQ ID: NO:1)).
- 1 5. The recombinant polynucleotide of claim 1 wherein the
2 heterologous nucleotide sequence codes for the expression of a transcription regulator,
3 interleukin-6, a tissue inhibitor of a metalloproteinase, collagen, elastin, a growth
4 regulatory transcription factor, an apoptosis regulator or a protein or RNA component of
5 telomerase.
- 1 6. The recombinant polynucleotide of claim 1 wherein the
2 heterologous nucleotide sequence codes for the expression of an inhibitor of collagenase,
3 an inhibitor of a metalloproteinase, a plasminogen activator, an inhibitor of a

4 plasminogen activator, interleukin-1 receptor antagonist, an insulin-like growth factors, a
5 cyclin or a cyclin-dependent kinase.

1 7. A method for expressing a heterologous nucleotide sequence in a
2 senescent cell comprising the step of providing the cell with a recombinant
3 polynucleotide comprising an expression control sequence operatively linked to a
4 heterologous nucleotide sequence, the expression control sequence comprising (1) a
5 senescence responsive element and (2) a heterologous minimal promoter positioned
6 downstream of the senescence responsive element, wherein the senescence responsive
7 element up-regulates transcription of the heterologous nucleotide sequence in a senescent
8 cell; and culturing the cell under conditions for expression.

1 8. An isolated recombinant host cell comprising a recombinant
2 polynucleotide comprising an expression control sequence operatively linked to a
3 heterologous nucleotide sequence, the expression control sequence comprising (1) a
4 senescence responsive element and (2) a heterologous minimal promoter positioned
5 downstream of the senescence responsive element, wherein the senescence responsive
6 element up-regulates transcription of the heterologous nucleotide sequence in a senescent
7 cell.

1 9. The recombinant host cell of claim 8 which is a fibroblast.

1 10. A transgenic non-human animal having a germ cell that comprises a
2 recombinant polynucleotide comprising an expression control sequence operatively linked
3 to a heterologous nucleotide sequence, the expression control sequence comprising (1) a
4 senescence responsive element and (2) a minimal promoter positioned downstream of the
5 senescence responsive element, wherein the senescence responsive element up-regulates
6 transcription of the heterologous nucleotide sequence in a senescent cell.

1 11. A method for monitoring senescence in a cell comprising the steps
2 of:
3 culturing, under conditions for expression, a recombinant host cell
4 comprising a recombinant polynucleotide comprising an expression control sequence

5 operatively linked to a heterologous nucleotide sequence encoding a reporter gene, the
6 expression control sequence comprising (1) a senescence responsive element and (2) a
7 minimal promoter positioned downstream of the senescence responsive element, wherein
8 the senescence responsive element up-regulates transcription of the heterologous
9 nucleotide sequence in a senescent cell;
10 measuring the amount of expression of the reporter gene by the
11 cell; and
12 determining whether the measured amount is different than the
13 amount expected in a senescent cell;
14 whereby the difference between the measured amount and the
15 expected amount reflects the degree to which the cell is senescent.

1 12. A method for determining whether a compound alters the activity
2 of a senescence responsive element in a cell comprising the steps of:
3 culturing, under conditions for expression; a recombinant host cell
4 comprising a recombinant polynucleotide comprising an expression control sequence
5 operatively linked to a heterologous nucleotide sequence encoding a reporter gene, the
6 expression control sequence comprising (1) a senescence responsive element and (2) a
7 minimal promoter positioned downstream of the senescence responsive element, wherein
8 the senescence responsive element up-regulates transcription of the heterologous
9 nucleotide sequence in a senescent cell;
10 contacting the cell with the compound;
11 measuring the amount of expression of the reporter polypeptide;
12 and
13 determining whether the measured amount is different than the
14 amount expected from a recombinant host cell to which no compound has been
15 administered;
16 whereby a difference between the measured amount and the
17 expected amount indicates that the compound alters the activity of the senescence
18 responsive element in the cell.

1 13. A compound identified by the method of claim 12.

1 14. A polynucleotide probe that specifically hybridizes with a
2 contiguous nucleotide sequence within nucleotides -35 to -134 of the interstitial
3 collagenase gene promoter, or with a sequence complementary to a contiguous nucleotide
4 sequence within nucleotides -35 to -134 of the promoter.

1 15. The polynucleotide probe of claim 14 further comprising a label.

1 16. An inhibitory polynucleotide comprising an antisense sequence of at
2 least 7 nucleotides that specifically hybridizes to a nucleotide sequence within nucleotides
3 -35 to -134 of the interstitial collagenase gene promoter, or the complement of the
4 promoter sequence, wherein the inhibitory polynucleotide inhibits transcription of
5 nucleotide sequences operatively linked to expression control sequences comprising a
6 senescence responsive element.

1 17. The inhibitory polynucleotide of claim 16 that specifically
2 hybridizes to a nucleotide sequence within nucleotides -89 to -66 of the interstitial
3 collagenase gene promoter, or the complement of the promoter sequence.

1 18. A method of inhibiting in a cell the expression of a nucleotide
2 sequence operatively linked to an expression control sequence that comprises a
3 senescence responsive element, the method comprising providing the cell with an
4 inhibitory polynucleotide comprising an antisense sequence of at least 7 nucleotides that
5 specifically hybridizes to a nucleotide sequence within nucleotides -35 to -134 of the
6 interstitial collagenase gene promoter, or the complement of the promoter sequence,
7 wherein the inhibitory polynucleotide inhibits transcription of nucleotide sequences
8 operatively linked to expression control sequences comprising a senescence responsive
9 element.

1 19. The method of claim 18 wherein the cell is an isolated recombinant
2 cell *in vitro*.

1 20. The method of claim 18 wherein the cell is a cell transfected *ex*
2 *vivo* and introduced into an animal.

- 1 21. A pharmaceutical composition comprising a pharmaceutically
2 acceptable carrier and a prophylactically or therapeutically effective amount of:
3 (a) a recombinant polynucleotide comprising an expression control sequence
4 operatively linked to a heterologous nucleotide sequence, the expression control sequence
5 comprising (1) a senescence responsive element and (2) a minimal promoter positioned
6 downstream of the senescence responsive element, wherein the senescence responsive
7 element up-regulates transcription of the heterologous nucleotide sequence in a senescent
8 cell;
9 (b) a recombinant polynucleotide comprising expression control sequences
10 operatively linked to a nucleotide sequence coding for the expression an inhibitory
11 polynucleotide comprising nucleotides in a sequence substantially identical to or
12 substantially complementary to a contiguous sequence of 6 nucleotides within nucleotides
13 -35 to -134 of SEQ ID NO:1, wherein the inhibitory polynucleotide inhibits transcription
14 of nucleotide sequences operatively linked to expression control sequences comprising the
15 senescence responsive element; or
16 (c) an inhibitory polynucleotide comprising an antisense sequence of at least 7
17 nucleotides that specifically hybridizes to a nucleotide sequence within nucleotides -35 to
18 -134 of the interstitial collagenase gene promoter, or the complement of the promoter
19 sequence, wherein the inhibitory polynucleotide inhibits transcription of nucleotide
20 sequences operatively linked to expression control sequences comprising a senescence
21 responsive element.

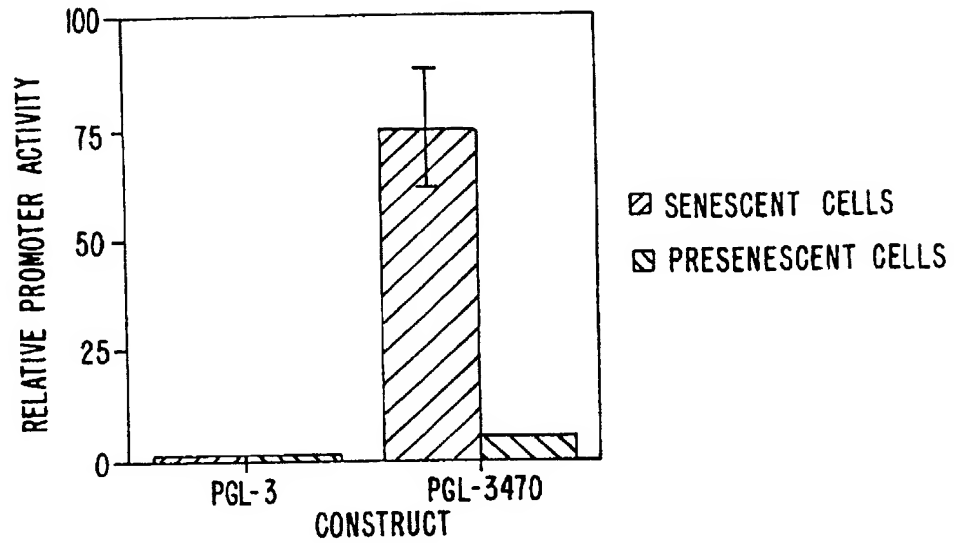
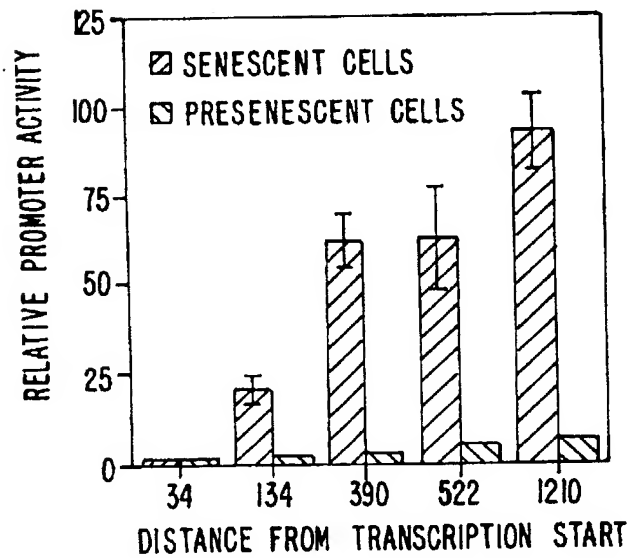
- 1 22. A method of prophylactically or therapeutically treating in a subject
2 a senescent condition associated with the activity of a senescence responsive element, the
3 method comprising the step of inhibiting the expression of a gene operatively linked with
4 an expression control sequence that includes the senescence responsive element, whereby
5 inhibiting expression of the gene provides a treatment of the senescent condition.

- 1 23. The method of claim 22 wherein the senescent condition is aged
2 epithelium, cancer or an inflammatory condition.

1 24. A method of prophylactically or therapeutically treating in a subject
2 a senescent condition associated with under-expression of a gene, the method comprising
3 the step of providing cells of the subject with a recombinant polynucleotide comprising
4 expression control sequences comprising a senescence responsive element operatively
5 linked with the gene, whereby expression of the gene provides a treatment of the
6 senescent condition.

1 25. A method of determining whether a sample comprises a compound
2 that binds to a senescence responsive element comprising contacting the sample with a
3 polynucleotide probe that specifically hybridizes with a nucleotide sequence within
4 nucleotides -35 to -134 of the interstitial collagenase gene promoter; and determining
5 whether a compound has bound to the probe; wherein binding indicates that the sample
6 contains a compound that binds to the senescence responsive element.

1/4

*FIG. 1.**FIG. 2.*

2/4




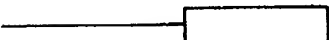
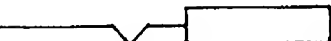
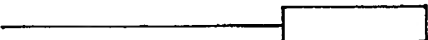
		FOLD ACTIVITY OVER MINIMAL PROMOTER	INCREMENTAL INCREASE
PGL-34		1.0	0
PGL-134		20.8	20.8
PGL-390		62.5	3
PGL-522		93.04	0.5
PGL-522 Δ 34/134		2.9	N/A
PGL-1210		92.8	N/A

FIG. 3.

Figure 4

Sequence of 100 bp Element Responsible for Collagenase Overexpression in Senescent Human Fibroblasts

(Position -134 to -34 in the human interstitial collagenase promoter)

Position -134 ↓ -120 ↓ -110 ↓
 5' AGTC ACCATTCTA ATGATTGCCT AGTCTATTCA

 -90 ↓ -70 ↓ -60 ↓
TAGCTAATCA AGAGGATGTT ATAAAGCATG AGTCAGACAC CTCTGGCTTT

 -40 ↓
CTGGAAGGGC AAGGACT 3'

5'-FLANK COLLAGENASE

FIG. 5.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/06504

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/85 C12Q1/68 G01N33/50 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C07K G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	WO 95 07993 A (ZENECA LTD) 23 March 1995 see the whole document	22 11,12, 14,16,18
X,P	--- WO 96 29858 A (WISCONSIN ALUMNI RES. FOUND) 3 October 1996 see abstract see page 4, line 25 - page 10, line 36 see page 20, line 10 - page 23, line 11 --- -/--	1,7,8,11

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
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- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

22 September 1997

Date of mailing of the international search report

07.10.97

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Authorized officer

Panzica, G

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US 97/06504

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EXPERIMENTAL GERONTOLOGY, vol. 29, no. 1, 1994, S.FRANCISCO, US, pages 37-53, XP002040658 BURKE E.M. ET AL.: "Altered transcriptional regulation of human interstitial collagenase in cultured skin fibroblasts from older donors" cited in the application	1-4,8,9
A	see abstract see page 37, paragraph 1 - page 39, line 2 see page 40, paragraph 2 - page 41, paragraph 1 see page 42, paragraph 1 - page 46, paragraph 1; figure 1	11,12
Y	---	1-4,8,9
A	WO 91 15226 A (AMERICAN NATIONAL RED CROSS) 17 October 1991 see the whole document	11,12, 14,16-19
Y	---	1-4,8,9
A	MOLECULAR AND CELLULAR BIOLOGY, vol. 7, no. 6, June 1987, WASHINGTON US, pages 2256-2266, XP002040659 ANGEL P. ET AL.: "12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region" cited in the application see the whole document	14,25
Y	---	1-4,11, 12
A	WO 95 06754 A (UNIV. CALIFORNIA) 9 March 1995 see abstract see page 7, line 144 - page 8, line 177 see claim 1	1-4
Y	---	11,12
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 2, 12 January 1996, MD US, pages 774-782, XP002040660 LOGAN S.K. ET AL.: "synergistic transcriptional activation of the tissue inhibitor of metalloproteinases-1 promoter via functional interaction of AP-1 and Ets-1 transcription factors" cited in the application see abstract see page 774, column 2, paragraph 2 - page 775, column 2, paragraph 3 see figure 2	1-4
Y	---	11,12
Y	US 5 491 069 A (DIRMI GOBERDHAN P. ET AL) 13 February 1996 see the whole document	11,12

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INTERNATIONAL SEARCH REPORT

International Applic: No
PCT/US 97/06504

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>EMBO JOURNAL, vol. 9, no. 7, 1990, EYNHAM, OXFORD GB, pages 2241-2246, XP002040661 GUTMAN A. AND WASYLYK B.: "the collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites" cited in the application see the whole document ---</p>	<p>1-4,8,9, 14,15,25</p>
A	<p>NUCLEIC ACID RESEARCH, vol. 20, no. 11, 1992, OXFORD, GB, pages 2699-2703, XP002040662 MAJERUS M.A. ET AL.: "Serum, AP-1 and Ets-1 stimulate the human ets-1 promoter" cited in the application -----</p>	<p>1-4</p>

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 97/06504

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Please see Further Information sheet enclosed.
2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
Please see Further Information sheet (Obscurities)
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA210

Remark: Although claims 22 - 24 are directed to a method of treatment of the human/animal body the search has been carried out on the basis of use and alleged effects of the subject matter.

Obscurities, inconsistencies, contradictions, lack of conciseness, lack of ready comprehensibility (EP: Art 84 EPC; SA, Art. 6 PCT)

Reason: Art. 6 PCT. Lack of disclosure.

Incomplete search:

Claims searched completely: 22 - 24

Claims searched incompletely: --

Claims not searched : 13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 97/06504

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9507993 A	23-03-95	AU 7619494 A CA 2172842 A EP 0719341 A	03-04-95 23-03-95 03-07-96
WO 9629858 A	03-10-96	AU 5020996 A CA 2191482 A	16-10-96 03-10-96
WO 9115226 A	17-10-91	AU 7676191 A CA 2080336 A EP 0527790 A	30-10-91 10-10-91 24-02-93
WO 9506754 A	09-03-95	AU 7719094 A CA 2169466 A EP 0724648 A JP 9502345 T NO 960715 A	22-03-95 09-03-95 07-08-96 11-03-97 22-02-96
US 5491069 A	13-02-96	NONE	